Spatial tropism profiling of AAV vectors by ultrasensitive sequential FISH in tissue

Ultrasensitive sequential fluorescence in situ hybridization (USeqFISH) enables multiplexed detection of the expression of endogenous and exogenous genes delivered by adeno-associated virus (AAV) vectors in intact tissue. USeqFISH provides a spatial map of AAV tropism with high throughput and resolution.

This is a summary of:
Jang, M. J. et al. Spatial transcriptomics for profiling the tropism of viral vectors in tissues. *Nat. Biotechnol*. https://doi.org/10.1038/s41587-022-01648-w (2023).

Published online: 30 January 2023

The problem

Adeno-associated virus (AAV) vectors have become a primary choice for delivering genes to biological systems. Although some natural AAV serotypes have undergone clinical trials (and a few are already approved[1]), efforts are focused on improving AAV vectors by engineering recombinant variants that enable efficient gene transfer, either broadly or preferentially to targeted organs or cell types, in various species, including rodents and non-human primates (NHPs), via minimally invasive routes[1]. The development of high-throughput selection platforms and single-cell sequencing-based characterization has accelerated the discovery of many promising vectors with potentially diversified tropism; however, the field has lacked an in-depth assay that can quantitatively score the efficiency of transduction and map the tropism of pooled AAV variants in situ. Here, we address this pressing need for a high-throughput, high-resolution technique for screening AAV tropism in tissues from biologically diverse systems.

The solution

We describe a method for ultrasensitive sequential fluorescence in situ hybridization (USeqFISH), which enables in-tissue, single-cell profiling of both endogenous mRNA and transcripts from barcoded AAV vectors (Fig. 1). Although many in situ spatial transcriptomics methods are available[2], they require a long barcode sequence (~0.5–1 kilobase (kb)), which poses a challenge for AAV vectors, as they can carry only a short genome (<4.7 kb). We addressed this technical hurdle by combining two signal amplification strategies, rolling circle amplification (RCA, in which a circular DNA is used to generate a chain of tandem repeats) and hybridization chain reaction (HCR, in which a pair of hairpins is used to form a self-assembly only when an initiator sequence is present). This approach enables the generation of highly sensitive RNA FISH signals by targeting a unique sequence of only 14 nucleotides (nt) in cell culture and 40 nt in tissue for selective visualization. Adapting a previously published two-step stripping method to detach fluorescent labels and an RNA-optimized passive CLARITY technique (PACT) for tissue clearing (making the samples penetrable and optically accessible)[3], we established a USeqFISH protocol that can sequentially detect up to ~50 genes in 50-µm-thick brain tissue of mice and NHPs.

We applied USeqFISH to profile the cell-type tropism in the mouse brain of six systemically delivered AAV capsids (the protein shell packaging the gene of interest), including variants we have previously described[4,5] and a new variant, AAV-PHP.AX. USeqFISH profiling confirmed our previous observations and, in addition, revealed distinct neuronal subtype biases of efficient variants. We found that AAV-PHP.AX exhibits robust transduction efficiency and broad coverage across neuronal subtypes and astrocytes, enabling us to effectively tune its tropism by pairing it with gene regulatory elements. We demonstrate the applicability of USeqFISH across mouse brain regions and its utility for profiling gene regulatory elements by using pooled microRNA target sites inserted in the AAV genome to selectively silence gene expression. Finally, we show the potential of USeqFISH for in situ profiling of AAV tropism and multimodal single-cell analysis in NHPs.

The implications

USeqFISH enables spatial transduction profiling of pooled AAV capsids and their cargoes (the sequences of interest), which we expect will expedite the development of precisely targeted, systemic gene delivery vectors. The method reveals distinct tropism biases for engineered capsids differing only in a few amino acids; such tropic capsids can enable the use of minimal gene regulatory elements (suitable for the limited genomic space of AAVs) and lower AAV doses for targeted gene delivery, leading to safer, more accessible genetic medicines. Importantly, USeqFISH will facilitate AAV engineering for NHPs by enabling multiplexed analysis of pooled AAVs economically in a single animal while bypassing the limited availability of cell-type-marker antibodies. Finally, USeqFISH can be combined with AAV tools for multimodal single-cell analysis.

One caveat of the current USeqFISH protocol is that the number of target genes and the maximum thickness of the tissue are limited. We expect that future adaptations of color barcoding methods and/or in situ sequencing strategies with tissue expansion will enable us to scale this method up to hundreds of variants in thicker tissue volumes. With these technical improvements, we expect USeqFISH to improve our understanding of AAV biology and expand the AAV toolkit for research and clinical applications.

Min Jee Jang & Viviana Gradinaru
California Institute of Technology, Pasadena, CA, USA.

EXPERT OPINION

"I think that USeqFISH with passive CLARITY technique can be useful to advance the field, especially if it indeed enables accurate detection with a single short probe. Overall, this paper includes new and useful data, which are presented in a clear way, and the new method introduced will find a general interest in the field." **Shahar Alon, Bar-Ilan University, Ramat Gan, Israel.**

FIGURE

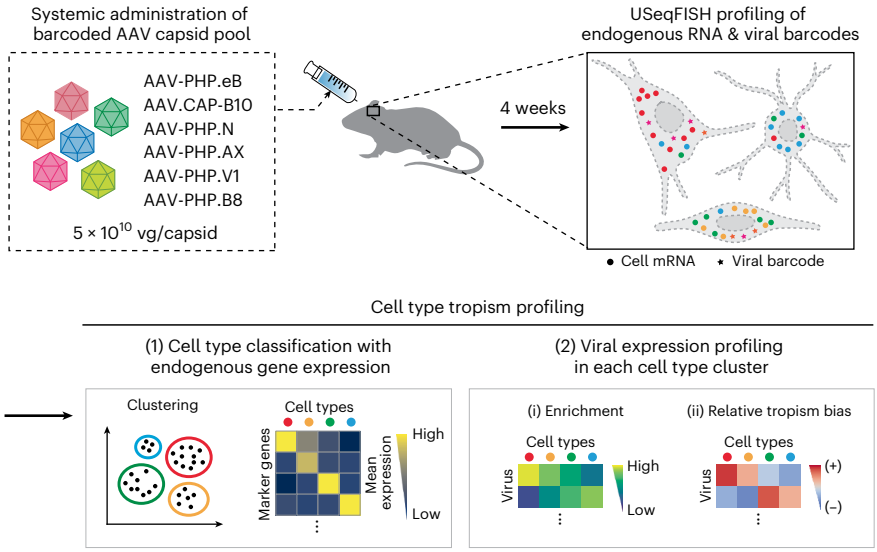

Fig. 1 | USeqFISH for in situ cell-type tropism profiling of pooled AAV vectors. A schematic of the procedure of USeqFISH profiling. vg, vector genomes.

BEHIND THE PAPER

As AAV engineers, we often must make difficult decisions when selecting which of many promising variants identified from library screening should be tested further. In most cases, our decisions have relied on enrichment of viral genomes measured from bulk sequencing, which could miss potentially useful variants with high cell-type specificity. Also, characterizing the cell-type specificity of the selected variants was limited by which antibodies were available. Thus, we initiated this project to address our (and the field's) need for quantitative methods to measure AAV transduction with single-cell resolution. Every step of the work, from designing and producing barcoded AAV pools to implementing computational pipelines for custom hardware control and data analysis, turned out to be a continual challenge due to COVID-19. Nevertheless, we are delighted to introduce this new method and thank all our contributors and collaborators for their help that made it happen. **M.J.J. & V.G.**

REFERENCES

1. Challis, R. C. et al. Adeno-associated virus toolkit to target diverse brain cells. *Annu. Rev. Neurosci.* **45**, 447–469 (2022).
   This review article presents the up-to-date status of AAV engineering for developing targeted gene delivery vectors.
2. Moses, L. & Pachter, L. Museum of spatial transcriptomics. *Nat. Methods* **19**, 534–546 (2022).
   This review article presents progress and applications of spatial transcriptomics technologies.
3. Yang, B. et al. Single-cell phenotyping within transparent intact tissue through whole-body clearing. *Cell* **158**, 945–958 (2014).
   This paper reports PACT for clearing intact organs while retaining RNA, making it compatible with FISH.
4. Goertsen, D. et al. AAV capsid variants with brain-wide transgene expression and decreased liver targeting after intravenous delivery in mouse and marmoset. *Nat. Neurosci.* **25**, 106–115 (2022).
   This paper reports an engineered AAV capsid that targets the brain and not the liver in mice and marmosets after systemic delivery.
5. Ravindra Kumar, S. et al. Multiplexed Cre-dependent selection yields systemic AAVs for targeting distinct brain cell types. *Nat. Methods* **17**, 541–550 (2020).
   This paper reports a highly multiplexed selection platform for Cre-dependent screening of AAVs, as well as a few capsids with specific cell-type tropism.

FROM THE EDITOR

"The authors profile both AAVs and endogenous RNA in intact tissues. This technique is important because it enables an improved characterization of cell types and subtypes targeted via AAV vectors and will enable improved targeting and gene delivery." **Editorial Team, *Nature Biotechnology*.**